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Seasonal Changes in Humoral Immunity and Blood Thyroxine Levels in the Toad, *Bufo regularis*

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ABSTRACT—The present study was designed to explore the basis of seasonal changes in the humoral immune response of the toad, *Bufo regularis*. The results indicated that; (1) administration of 0.4 ml of 10% RRBC suspension during the breeding season and the time of burrowing for winter elicited a high titer of antibody (Ab) and vigorous rosette-forming cell (RFC) resoponse. In contrast, during summer active life period and hibernation, immune response was slow with a low titer of Ab and minimal number of RFC; (2) cold-acclimated toads (8–10°C; 3 weeks) provided a considerable amount of serum Ab in response to primary immunization with RRBC; despite that the magnitude and the kinetic of the response were significantly different from those of control toads; (3) fluctuation in thyroxine (T_4) levels was in direct correlation with the immunological indices recorded for adult toads during the different periods of the year. Our results suggest that endogenous T_4 levels generally trigger *B. regularis* immune system with low levels causing a significant inhibition and with high levels causing a significant stimulation of humoral immune response.

INTRODUCTION

Ectothermic vertebrates have a variable body temperature and each has its own thermal tolerance range, in which its life process can normally operate. As environmental temperature changes with season, each species acclimates and responds adaptively to the thermal range [1–3]. The literature dealing with seasonal rhythms in amphibian immunology appears to be scant. Special emphasis was focused on seasonal changes in the thymus architecture, in relation to the annual changes in animal activity [4–6]. That is, a maximum thymic weight is recorded during the summer active months. A marked involution occurs in winter, and is followed by gradual recovery of this organ, and this in turn is terminated in the mating season. Until recently little attention has been directed towards seasonal effects on immune response of amphibians.

Although, most authors reported some temperature-dependent immunodepression during winter, results were contradictory, and the causative agents poorly defined [for review see 1]. Recently, other authors have pointed out a possible role of endocrine modulators [5, 7]. Thyroid hormones, mainly thyroxine (T_4), experienced seasonal variations, dramatically affecting physiological activities in amphibians [8, 9]. Little is known, however, about their effects on the immune system. Therefore, the present study aimed at contributing information about some biological variables such as season and temperature on the humoral immune response of the toad, *Bufo regularis*. The outcoming results were particularly discussed from the plausible role played by T_4 levels in immunity of toads.

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MATERIALS AND METHODS

Toads

Adult male and female toads, *Bufo regularis*, were collected from Abu Rowash area (Egypt). A total of 700 toads weighing between 18–20 g and of length 8–9.5 cm long from snout to vent, were used in the present study. Toads were placed in glass aquaria with tap water. Every two days granulate trout feed were given *ad libitum*. Ani-

mals were maintained in a sunny animals room under natural light and ambient temperature of 10–17°C in winter, 18–27°C in spring and autumn and 30–38°C in summer. The amphibian life cycle in Egypt was demarcated into: (1) the breeding season (April-May), (2) the summer active life (June-September), (3) the time of burrowing for winter (October-November), and (4) the hibernation (December-February).

Preparation of spleen cells

Spleen was excised from individual toads and placed in separate petri-dishes in ice-cold buffered-amphibian saline (BAS), pH=7.2. Monocellular suspensions were prepared, cells were washed twice by centrifugation at $150\times g$ for 5 min, and pellets were resuspended in known volumes of BAS. Lymphocytes were counted and their viability was assessed by the trypan blue exclusion.

Antigen and immunization

Blood was collected from at least two or three healthy animals [rats (RRBC) or guinea pigs GRBC)] by either decapitaion or by heart puncture. Pooled blood was mixed with an equal volume of heparinized phosphate-buffered saline (PBS), pH=7.2, and centrifuged at $150\times g$ for 15 min. Cells were washed three times with ice-chilled PBS, pH=7.2. Toads were allowed to acclimate to ambient temperature in the laboratory for a few days before immunization. Preliminary experiments were carried out to determine the optimum dosage and effective route of immunization. From these experiments, 0.4 ml of 10% RRBC was found to be an optimum dosage which induced maximum antibody response when given intraperitoneally (i.p.). This immunization schedule was followed to study the kinetics of antibody response. Control unimmunized toads were injected i.p. with 0.4 ml PBS, pH=7.2 and included in each experiment.

Bleeding and serum preparation

Individual blood samples were allowed to clot for 2 hr at room temperature then overnight at 4°C. After centrifugation at $250\times g$ for 15 min, individual sera were immediately used or stored at 20°C. Immunized and unimmunized control sera were decomplemented at 56°C for 30 min before testing to eliminate spontaneous hemolytic factors and homologous complement activity.

Immunocytoadherence assay (ICA)

The immunocytoadherence (ICA) assay was essentially as described by Kidder *et al.* [10] with minor modification. Briefly, $1\text{–}2\times10^6$ viable spleen cells were separately mixed with 10×10^6 RRBC in a final volume of 100 μl culture medium in a serological glass tube. Culture medium was prepared by mixing L-15 medium (GIBCO, Grand Island, N.Y. U.S.A.) with heat-inactivated fetal calf serum in the ratio of 9:1. The medium was found to be optimal for the viability of toad cells and the maintenance of RRBC intact. Tubes were incubated for 15 min at 37°C and then overnight at 4°C. After resuspending the reaction mixture by gentle rotation and adding one drop of trypan blue, ICA-positive cells (rosettes) and lymphocytes were counted in a haemocytometer at a magnification of 250×. Each tube was counted once or twice depending on the variation among them. An ICA-positive cell (rosette-forming cell or RFC) was defined as a "rosette" consisting of a spleen cell bearing at least three adherent RRBC. Multiple layered rosettes with lymphocytes seen clearly at the center were considered as positive. No dead cells were found to form rosette. Two trails of each sample tested were run and the data were expressed as RFC/10^6 spleen cells. In order to determine the specificity of "rosette" formation, RRBC was replaced by GRBC in the assay system.

Haemagglutination (HA) assay

Circulating antibody (Ab) titers were determined according to standard procedure in microtitration plates as described in details previously [11]. Briefly, 100 μl two-fold diluted immune or control sera in PBS, pH=7.2 and 50 μl of 1% RRBC in PBS were mixed in 96 well of round-bottom microtiter plates (Nunc, Roskid, Denmark). After gentle aspiration, plates were incubated at 4°C and read 24 hr later. Titers were then recorded as the log of the last well showing microscopic agglutination, the first well had a final dilution of 1:2. Control wells contained PBS and erythrocytes only.

Radioimmunoassay of serum T_4

Determination of T_4 was carried out by a modification of the solid phase technique described in detail by Murphy *et al.* [12]. The antiserum used was raised in rabbit against L-thyroxine and supplied by Kallested Laboratories (Texas, U.S.A.). This antiserum showed a nearly 100% crossreactivity with T_4, 2.78% with triiodothyronine, but less than 0.01% with diiodothyronine as provided with the radioassay manufacture protocol. In this system, serum samples require neither extraction nor predilution. Serum aliquots (20 μl) were pipetted directly to the bottom of the corresponding assay tubes. Two hundred microliters of ^{125}I-T_4 were added to each tube and agitated on vortex mixer for one min. After one hr incubation at room temperature, separation of free T_4 from bound was accomplished simply by decanting the supernatant. The assay tubes were allowed to drain and then were inverted for 2 min on absorbent paper to shake off all the residual droplets. The tubes were thereafter counted for one min in gamma counter apparatus (Mini. Irst. Ltd., Essex, U.K.). Replicate analysis of a sample of toad serum gave intra-assay coefficient of variation of 2.6% and inter-assay coefficient of variation of 4.6%. Aliquots of serum were assayed and the smallest amounts of T_4 statistically distinguishable from zero was 1.0 μg/dl.

Statistical analysis

Student's t-test was used to determine levels of significance between control and experimental groups. Differences were considered to be significant when P values <0.05 were obtained.

RESULTS

Season-related differences in humoral immunity

The objective of this experiment was to evaluate the humoral response of adult toads during their annual life cycle. Data of two seprate experiments performed in sequence for each period, were similar, and therefore pooled (Figs. 1 and 2).

As depicted in Fig. 1, during hibernation period, the number of RFC rose quickly with low level on day 4, decreased sharply by day 8 and remained at this level until day 16. In active life period, immunized toads exhibited a peak at day 4, then sharply declined at day 8 and remained constant during the following eight days. However, in the time of burrowing for winter, response was significantly vigorous. High level of RFC was detected on day 4 post-immunization. Thereafter, the number of RFC began to decline but remained at high level up to day 16. In breeding season, the kinetics of RFC was similar to that demonstrated during the time of burrowing for winter. Indeed RFC were detectable at high level after day 4, peak was attained on day 12 followed by gradual decline (Fig. 1).

The kinetics of anti-RRBC Ab response is depicted in Fig. 2. During hibernation, Ab titer rose

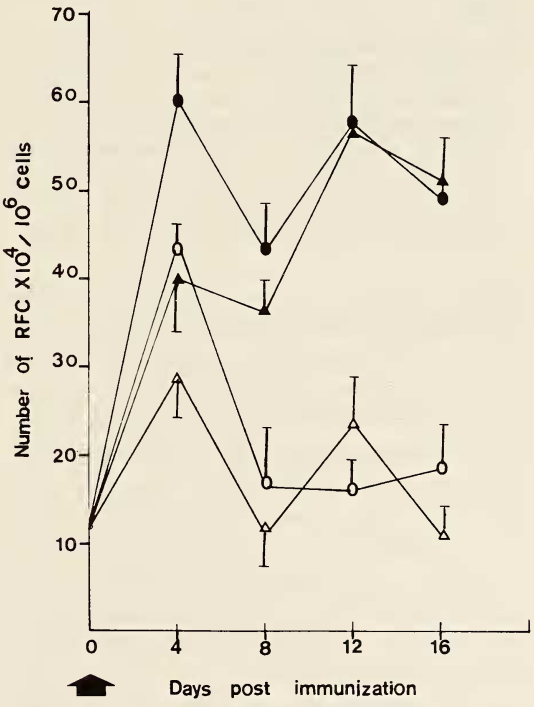

FIG. 1. Kinetics of rosette-forming cell (RFC) response in the spleen of adult toads, *B. regularis*. Animals were immunized on day 0 with 0.4 ml of 10% RRBC suspension in the breeding season (◀—▶), the active summer life (○—○), the time of burrowing for winter (●—●) and the hibernation (◁—▷). Each point represents the mean response of 3–5 separate animals, and the vertical bars indicate standard error of the mean.

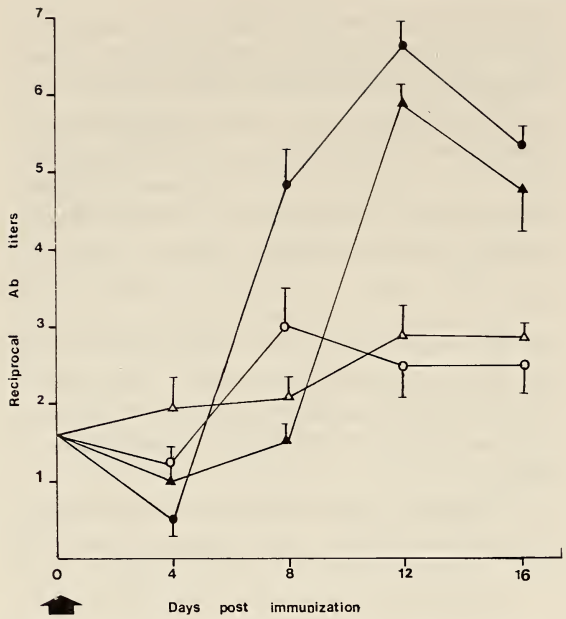

Fig. 2. Serum haemagglutinin titers in adult toads, *B. regularis*. Animals were immunized on day 0 with 0.4 ml of 10% RRBC suspension in the breeding season (◀—▶), the active summer life (○—○), the time of burrowing for winter (●—●) and the hibernation (◁—▷). Each point represents the mean response of 3–5 separate animals, and the vertical bars indicate standard error of the mean.

rapidly with low level of serum Ab activity on day 4, and almost remained constant until day 12. The titer increased to reach the low detectable level on day 16. In summer active life period, immunized toads exhibited a slight rise in serum HA titer at day 8 post-immunization and remained constant during the following eight days. However, in the time of burrowing for winter, Ab was first detected day 8 post-immunization and titer increased sharply reaching a maximum level on day 16. In the breeding season, the kinetics of Ab response was quite similar to that detected during the time of burrowing for winter (Fig. 2).

In summary, the results indicated that, during hibernation and summer active life period, humoral and cellular response was minimal with a small amount of RFC and low level of circulating Ab. In contrast, administration of RRBC (0.4 ml; 10% RRBC suspension) during the time of burrowing for winter and breeding season elicited a high titer of circulating Ab and vigorous FRC response.

Effect of cold acclimation on humoral immunity

The toads, field-collected in mid August, were immediately used for this experiment. About 25 toads were injected i.p. with 0.4 ml of 10% RRBC and maintained in the laboratory at ambient temperature (control group). Another group of toads was transferred to the dark incubator and kept without feeding at the temperature of 8–10°C for three weeks. Then, cold-acclimated toads were injected i.p. with 0.4 ml of 10% RRBC (experimental group).

As depicted in Fig. 3, control toads exhibited a peak at day 4, sharply declined at day 8 and remained constant during the following days.

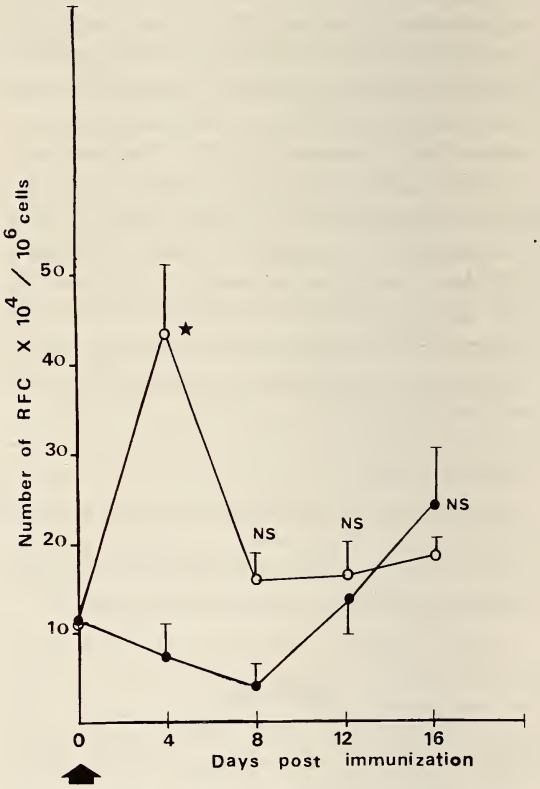

Fig. 3. Kinetics of RFC response in the spleen of adult toads, *B. regularis*. Fresh, field-collected (○—○) and cold-acclimated (●—●) animals were immunized on day 0 with 0.4 ml of 10% RRBC suspension. Each point represents the mean response of 3–5 separate animals, and the vertical bars indicate standard error of the mean. $*=0.05<P<0.01$, NS =not significant.

However, in experimental toads, low number of RFC was detected below background level on day 8. Therefore, the response gradually increased.

As depicted in Fig. 4, in control toads, Ab titers increased from day 4 reaching a peak level on day 8 and remained constant until day 12. Therefore, Ab titer sharply declined by day 16. In experimental toads, however, the kinetics of Ab response was not quite similar to that detected in control toads. Ab levels increased gradually, reaching its peak level on day 8, sharply dropped by day 12, then increased again.

Conclusively, primary immune response of cold-acclimated toads was significantly different from that of control toads kept at ambient temperature.

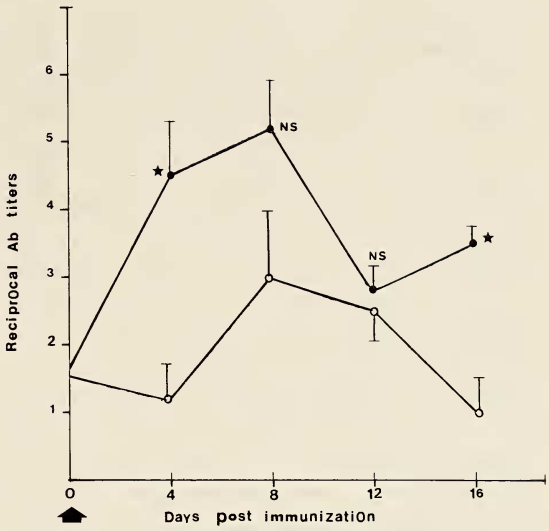

FIG. 4. Serum haemagglutinin titers in adult toads, *B. regularis*. Fresh, field-collected (○—○) and cold-acclimated (●—●) animals were immunized on day 0 with 0.4 ml of 10% RRBC suspension. Each point represents the mean response of 3–5 separate animals, and the vertical bars indicate standard error of the mean. $*=0.05<P<0.01$, NS=not significant.

Seasonal distribution of splenic lymphocytes

Since the difference of *B. regularis* lymphocytes to respond in humoral immunity could be due to significant differences in the number of lymphocytes or lymphocyte subsets, splenic lymphocytes of male and female toads were enumerated by the

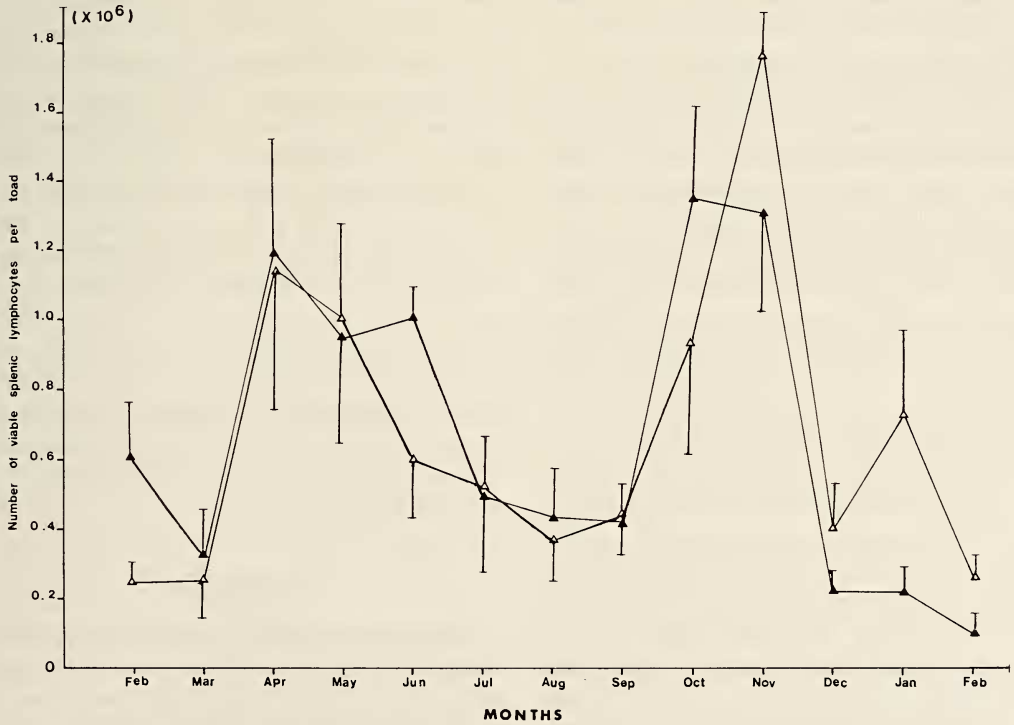

FIG. 5. Number of viable splenic lymphocytes obtained from female (◁—▷) and male (◀—▶) adult toads, *B. regularis* during the different months of the year. Each point on the curve expresses mean value of 10–15 separate animals and the vertical bars indicate standard error of the mean.

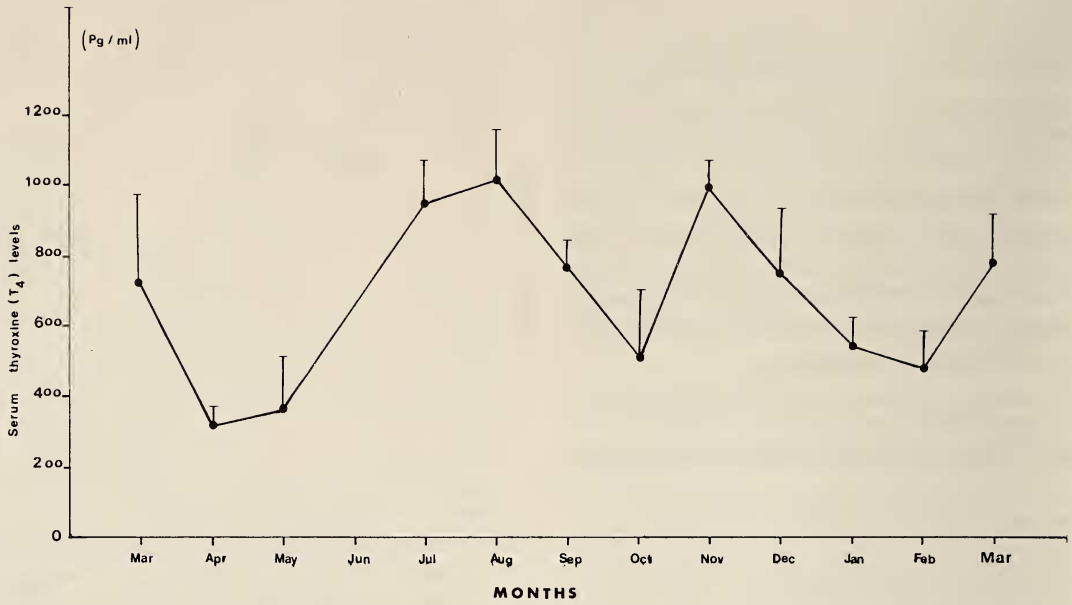

Fig. 6. The levels of the serum thyroxine of adult toads, *B. regularis* throughout the year. Each point on the curve represents the mean level of 8–10 samples and the vertical bars indicate standard error of the mean.

trypan-blue exclusion test. Determinations were performed on healthy, fresh field-collected animals. Three to five toads/sex groups were sacrificed at 10-day intervals throughout the year.

As depicted in Fig. 5, no statistically significant variation in the number of splenic lymphocytes could be observed between male and female toads. However, data obtained on toads exposed to natural experimental conditions suggest that a seasonal rhythm in lymphocyte distribution is clearly present. Viable splenic lymphocytes were more abandant in April-June and again in October-November. Minimal levels were observed during July-September and during December-March.

Seasonal rhythms in endogenous T_4 level

The monthly changes in circulating T_4 levels were assayed at 10-day intervals throughout the year. Determination was performed on serum samples from healthy fresh, field-collected toads. It is worth mentioning that these toads were from the same batches used throughout the experimental immunological studies. Preliminary determination failed to show sex-related differences in T_4 levels between male and female toads, and therefore the results were pooled.

As depicted in Fig. 6, T_4 exhibits monthly variation between 320 and 1006 pg/ml throughout the year. From April to May, serum concentration was around 320–360 pg/ml and increases up to 940 $\pm$139 pg/ml in August. From August through October, the serum levels of T_4 exhibited a sudden decrease. T_4 levels began to rise up to 933$\pm$33 pg/ml in November. From December, a precipitous decrease occurred in T_4 level reaching a basal value of 540$\pm$12 pg/ml in February, followed by a significant increase in March around 780$\pm$43 pg/ml.

The present data indicated that serum T_4 level was greatest in July-September (the active life period) and November (the time of burrowing for winter). Minimal levels were observed in January-February (the hibernation) and April-May (the breeding season).

DISCUSSION

The toad, *B. regularis,* is terrestrial, except for a short period during breeding season. The annual life cycle of the adult toads of this species consists of several distinct phases: the breeding season, active terrestrial life after breeding season, autum-

nal migration and hibernation [14]. Our results indicated that, administration of 0.4 ml of 10% RRBC suspension during the breeding season and the time of burrowing for winter elicited high titer of circulating antibodies and vigorous RFC response. In contrast, during both the summer active life period and hibernation, the humoral response was slow, with a low titer of circulating antibodies and limited number of RFC. Our results suggesting that seasonal rhythms strongly influenced the kinetics have invited into a study whether, in *B. regularis,* splenic lymphoid compartments are also affected by seasonal rhythms. Lymphocyte density changed in the spleen throughout the year. However, no sex-related difference was observed.

Various factors, principally temperature, implicated in seasonal variations affected the immune reactivity of amphibians and other ectotherms [1–3]. Immunosuppressive effects of winter were repeatedly related to low temperature. However, the environmental temperature failed to explain satisfactorily the differences in the immune response in the breeding season and summer active life reported herein for *B. regularis,* since the natural temperature is high in both periods. Indeed, the immune response was significantly different between animals collected during the time of burrowing for winter and hibernation; although ambient temperature is relatively low in Egypt in both periods. The data also indicated that cold-acclimated toads (8–10°C; 3 weeks) produced a considerable amount of serum antibodies in response to primary immunization with RRBC. In this respect, temperature was not the sole causative factor that affected seasonal rhythms in the toad's immune response. In agreement with our observations, Bigaj and Plytycz [4] have proved that experimental changes of the external temperature, which demand unphysiological behaviour of experimental frogs, cannot change significantly the season-specific morphology (and probably the function) of their thymus glands.

Therefore, other factors have been suggested as potential cause of seasonal changes affecting the immune system of amphibians. We think, however, that numerous physiological parameters regulate the seasonal variations of amphibian immune system. The correlation of the latter with T_4 seasonal fluctuation might attributed a role of the thyroid hormones in this scenario. The present study, performed to test this hypothesis, apparently gave T_4 a relatively important role. Serum T_4 levels were rather low in the breeding season and hibernation period, while the time of burrowing for winter (only during November), prebreeding and active summer life period were associated with a remarkable elevation in endogenous T_4 levels.

The moderate or profound decrease in lymphoid density and immune response observed during the prebreeding, active summer life and hibernation are in consequence to low endogenous T_4 level during these periods. On the other hand, enrichment of lymphoid cells and powerful immune response observed during the breeding and the time of burrowing for winter are in consequence to high endogenous T_4 levels.

Informations concerning the influence of T_4 on the immune system of amphibians is scant. It was known that in amphibians T_4 plays a fundamental role in triggering metamorphosis itself and many, if not all, regressive and progressive changes [15, 16]. The levels of T_3 and T_4 in the serum increased rapidly reaching their peaks when metamorphosis was at full swing [17]. Metamorphic events could be dissected by thyroid hormone concentration. However, no absolute threshold of induction exists and any concentration of T_4 could induce anuran metamorphic changes [18]. Rivier and Cooper [19] reported that injection of excessive T_4 into larvae of *Rana catesbeiana* resulted in regression of larval lymphoid organs. However, Bovbjerg [20] and Nagata [21] reported that modulation of T_4 levels during metamorphosis with thiourea or exogenous T_4 did not affect changes in allograft rejection. Since the inhibition of allograft rejection by metamorphosis depends on the genetic relatedness of donor and host [22], it is difficult to interpret these findings, since genetically heterogeneous populations were used. Moreover, the timing of hormonal variation might be crucial. Lastly, in young adults of *Xenopus laevis,* 10^{-7} M T_4 *in vitro* could affect antigen recognition and binding capacity of SRBC-sensitized splenic lymphicytes [18].

Our present observation might help in interpreting the amphibian data by suggesting that T_4 will

differentially stimulate and/or inhibit the immune system, depending on the time duration during which this hormone is released. We think that the correlation between the immune system and T_4 levels in our present data is not a mere coincidence in time but rather one of the arms of a dynamic immunoendocrine mechanism at work in ectothermic animals.

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